Journal of Medical Virology 56:58–65 (1998)

Viral Proteins VP2, VP6, and NSP2 Are Strongly Precipitated by Serum and Fecal Antibodies From Children With Rotavirus Symptomatic Infection

Javier Colomina,[1] Maria Teresa Gil,[1] Pilar Codoñer,[2] and Javier Buesa[1]*

[1]*Department of Microbiology, Hospital Clinico Universitario, School of Medicine, University of Valencia, Valencia, Spain*

[2]*Department of Pediatrics, Hospital Dr. Peset, Valencia, Spain*

Rotavirus-specific IgA has been correlated with immune protection against rotavirus reinfection and symptomatic disease. Systemic and mucosal antibody responses were determined by an enzyme-linked immunosorbent assay in 11 infants with severe rotavirus gastroenteritis. Geometric mean titers of antirotavirus serum IgG and IgA antibodies were significantly higher during the convalescence of the disease ($P < 0.001$ vs. acute-phase titers). Rotavirus-specific fecal sIgA antibodies increased 4 times during the convalescence in 9 (81.8%) children ($P < 0.001$). The serum IgG and IgA antibody and fecal sIgA antibody responses to individual rotavirus polypeptides were characterized by radioimmunoprecipitation assay (RIPA) using *Staphylococcus aureus* protein A and the lectin jacalin to precipitate IgG- and IgA-immune complexes, respectively. The main IgG response was directed toward the structural viral proteins VP2, VP4, and VP6 and toward the nonstructural protein NSP2. Serum IgA reactivity was detected by RIPA in all serum samples, with major responses to VP2, VP6, and NSP2. Interestingly, fecal sIgA in convalescent samples reacted strongly toward NSP2 and VP6. These data reinforce the antigenic importance of rotaviral proteins other than VP4 and VP7, such as VP2, VP6, and NSP2, as main targets in the immune response to rotavirus. ***J. Med. Virol. 56:58–65, 1998.***

KEY WORDS: rotavirus gastroenteritis; immune response; radioimmunoprecipitation; viral antigens; IgA; coproantibodies

INTRODUCTION

Rotaviruses are the main etiologic agents of severe gastroenteritis in infants and young children. It has been estimated that they infect over 90% of humans by three years of age [Kapikian and Chanock, 1996], causing almost one million deaths worldwide each year [Glass et al., 1994]. Development of rotavirus vaccines is an urgent need for preventing severe diarrhea, which leads to dehydration and death, and several candidate vaccines are currently being evaluated [Midthun and Kapikian, 1996]. Despite extensive research on rotaviruses, the precise nature of the immunological effector mechanisms involved in protection against rotavirus disease are only partly understood [Ward, 1996]; complete knowledge is needed to design an efficient vaccine strategy. The rotavirus outer capsid proteins VP4 and VP7 induce neutralizing antibodies that protect experimental animals in vivo, but their relative importance in protective immunity in humans is not known. Several animal studies have shown that local intestinal antibodies rather than circulating serum antibodies play an important role in the protective process [Offit and Clark, 1985; Feng et al., 1994]. Therefore, analysis of mucosal immune responses to rotavirus antigens is of major importance for a better understanding of the protein-specific immune response after naturally acquired rotavirus infections.

A variety of serological assays have been developed to determine the levels of rotavirus-specific antibodies [Kapikian and Chanock, 1996]. However, only studies performed by radioimmunoprecipitation (RIP) assays favor the detection of antibodies directed against most rotavirus-specific structural and nonstructural proteins [Offit et al., 1983; Svensson et al., 1987].

Rotavirus-specific human IgM, IgG and IgA antibodies have been reported in serum as well as in local secretions (saliva, duodenal fluid, and fecal material)

Contract grant sponsor: the Fondo de Investigaciones Sanitarias (F.I.S.) of the Spanish Ministry of Health; Contract grant number: 96/1173.

*Correspondence to: Dr. Javier Buesa, Department of Microbiology, School of Medicine, University of Valencia, Avda. Blasco Ibañez 17, 46010 Valencia, Spain. E-mail: javier.buesa@uv.es

Accepted 4 March 1998

[Grimwood et al., 1988; Coulson et al., 1989]. Persistence of serum IgA rotavirus antibodies after natural infections has been observed for at least six months in humans and longer in mice [Grimwood et al., 1988; Shaw et al., 1993]. Preexisting rotavirus-specific IgA rather than IgG in serum has been found to correlate with milder symptoms in young children exposed to natural rotavirus infection [Hjelt et al., 1987; Feng et al., 1994; Johansen et al., 1994]. It has been established in children that after natural infection, as well as after oral vaccination, the structural proteins VP2, VP4, and VP6 are the immunodominant polypeptides recognized by IgG antibodies [Svensson et al., 1987; Richardson et al., 1993]. In experimental rotavirus infections of adult mice, VP6 and VP4 have been found to be the most immunogenic proteins in both sera and stools [Ishida et al., 1996].

Jacalin, a D-Gal binding lectin obtained from the jackfruit *Artrocarpus integrifolia,* has been reported to bind specifically to human immunoglobulin serum and secretory IgA (subclass IgA1), and has been used to separate this immunoglobulin from IgG and IgM [Roque-Barreira et al., 1985; Johansen et al., 1994].

In this article we measure the serum IgM, IgG, IgA, and fecal secretory IgA (sIgA) immune responses to rotavirus by enzyme immunoassay and we analyze the polypeptide specificity of serum IgG, IgA, and fecal sIgA to individual rotavirus proteins by RIP assay in children with severe acute rotavirus gastroenteritis.

MATERIALS AND METHODS

Patients and Specimens

The samples analyzed in the present study were collected from 11 patients (5 males; 6 females) aged between 2 and 24 months (average age 10.8 months). The children were hospitalized with acute rotavirus diarrhea and all infections were diagnosed within a few hours of admission by enzyme immunoassay (Rotaclone, Cambridge Biotech, Worcester, MA) and electron microscopy of negatively stained fecal suspensions concentrated by ultracentrifugation (100,000 g for 1 h).

Stool and blood specimens were collected from each patient. Acute-phase samples were obtained within 72 hours after the onset of diarrhea and convalescent-phase samples were obtained 18–33 days after the onset of symptoms. Blood samples were allowed to clot at room temperature and centrifuged at 750 g for 5 min. Ten percent (w/v) fecal suspensions were made in TNC buffer (10-mM Tris-HCl (pH 7.4), 100-mM NaCl, 1-mM $CaCl_2$).

Cells and Viruses

Confluent monolayers of fetal rhesus monkey kidney cells MA104 in 75-cm^2 flasks (Costar, Cambridge, MA) were washed twice with phosphate-buffered saline (PBS) and infected with trypsin-activated (type IX, Sigma, St. Louis, MO) simian rotavirus SA11 (serotype G3, P[2]) at a multiplicity of infection (m.o.i.) of 1 or mock-infected. After 1-hour adsorption at 37°C, the virus inoculum was washed off and Eagle's minimal essential medium (EMEM, Gibco-BRL, Paisley, U.K.) containing 1-μg/ml trypsin without serum was added. The cells were harvested when a cytopathic effect extending to >90% of the cell monolayer was evident. CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT) and used to propagate a recombinant vaccinia virus expressing the VP7 protein from the RF strain of bovine rotavirus provided by J. Cohen of INRA, Jouy-en-Josas, France.

The VP7 G-serotypes of the infecting rotavirus strains were determined by ELISA with type-specific monoclonal antibodies as described [Coulson et al., 1987].

Concentration and Purification of Virus

Rotavirus-infected MA104 cells were disrupted by three cycles of freezing and thawing, and the resultant suspension clarified by centrifugation for 25 min at 15,000 g. The cell pellet was resuspended in TNC buffer and extracted twice with trichloro-trifluoroethane (Serva, Heidelberg, Germany). Purification of virus was performed from the aqueous phase by isopycnic centrifugation in a CsCl gradient as previously described [Offit et al., 1983]. The concentration of viral proteins was determined by the Bradford assay (Bio-Rad Protein Assay, BioRad, Hercules, CA).

Enzyme Immunoassays

Rotavirus-specific IgM, IgG, IgA, and sIgA antibodies in paired acute and convalescent samples were detected by ELISA as described [Inouye et al., 1984; Coulson et al.,1989]. Briefly, purified viral antigen preparations were treated with 1.5-M NaSCN, incubated at room temperature for 15 min, and diluted with 0.06-M carbonate-bicarbonate buffer (pH 9.6) to a final concentration of 2 μg/ml. Volumes (100 μl) of this antigen preparation were dispensed into wells of microtiter plates (Nunc Immuno I, Nunc, Roskilde, Denmark). Serum samples were serially diluted with PBS–0.1% Tween 20 (PBS-T) containing 1% BSA, starting from 1/50 for IgM and IgG and from 1/25 for IgA, and added to antigen-coated wells. Ten percent (w/v) fecal suspensions, regarded as 1:10 dilutions, were further diluted in PBS-T. After incubation at 37°C for 2 hr, plates were washed and peroxidase-conjugated goat antihuman IgM, IgG, or IgA (Sigma Immunochemicals, St. Louis, MO) in 1% BSA-PBS-T were added and incubated for 2 hr at 37°C. After three washes, the reactions were developed using 3,3′-5,5′-tetramethylbenzidine (Sigma Immunochemicals) and stopped with 0.5-M H_2SO_4. Optical density (OD) was determined at 450 nm. Optimal dilutions of reagents were determined by checkerboard titration. Paired serum samples from each child were tested on the same plate. A sample was considered to be positive when the OD was greater than or equal to three times the negative control (mock-infected MA104 cells).

Radiolabeling of Rotavirus Polypeptides

Confluent monolayers of MA104 cells in 75-cm^2 flasks (Costar) were washed twice with PBS and incubated with EMEM without serum 12 hours before infection. The cells were either infected with trypsin-activated SA11 rotavirus (5 m.o.i.) or mock-infected. After 1-hour adsorption at 37°C, the inoculum was removed and EMEM supplemented with 1-μg/ml trypsin and 3-μg/ml actinomycin D (Cosmegen, MSD, West Point, PA) was added. At 8-hour postinfection, the medium was replaced with methionine- and cysteine-free EMEM (Sigma), equally supplemented with trypsin and actinomycin D, containing 10-mM HEPES. At 9-hour postinfection, the cells were labeled with 100-μCi/ml [^{35}S]methionine (ICN Biomedicals Inc., Costa Mesa, CA) for 3 hours at 37°C. The supernatant was then removed and cells rinsed twice with PBS before lysis with 1-ml RIPA-buffer (10-mM Tris-HCl [pH 7.8], 150-mM NaCl, 600-mM KCl, 5-mM EDTA, 2% Triton X-100, 1-mM phenylmethylsulfonyl fluoride, 1-μg/ml aprotinin) for 30 min on ice. The lysates were then centrifuged for 30 min at 30,000 g (5°C) and the supernatants stored at –70°C and used as a source of labeled rotavirus proteins for RIP assays.

IgG-, IgA-, and sIgA-RIP Assays

Cell lysates were preadsorbed with equal volumes of 10% *S. aureus* protein A Cowan I suspension (Sigma) or jacalin-agarose (Sigma) for the detection of IgG or IgA class antibodies, respectively. After incubation for 1 hr at 4°C, the supernatant was collected by centrifugation for 10 min at 30,000 g. RIP assays were performed by mixing 10 μl of undiluted serum samples (in serum IgG- and IgA-RIP assays) and 10 μl of 10% fecal suspensions (in fecal IgA-RIP assays) with 10 μl of radiolabeled rotavirus or mock-infected preadsorbed cell lysate in a total volume of 100 μl RIPA buffer. After overnight incubation at 4°C, 50 μl of 10% *S. aureus* protein A for the IgG assay and 30 μl of jacalin-agarose for the IgA and sIgA assays were added to the mixtures and incubated for 1 hr at 4°C with frequent vortexing. The precipitated immune complexes were washed three times with 0.15-M NaCl, 1-mM EDTA, 0.25% BSA, 0.05% Triton X-100, 0.02-M sodium azide in 0.05-M Tris-HCl (pH 7.4). The adsorbed labeled proteins were recovered by resuspending the pellets in 30 μl of sample buffer containing 2% SDS, 5% 2-mercaptoethanol, 20% glycerol, 62-mM Tris-HCl (pH 6.8), and 0.01% bromophenol blue. Immune complexes were dissociated by boiling the suspensions for 5 min and applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and Fluorography

Samples were analyzed by SDS-PAGE using a discontinuous Tris-glycine buffer system. Immune complexes were electrophoresed at 15 mA for 45 min. Molecular weight markers were included (prestained SDS-PAGE Standard, Broad Range, BioRad). Gels were fixed for 30 min in 10% glacial acetic acid, 35% methanol, soaked in Amplify (Amersham Life Science, Little Chalfont, U.K.) for 30 min, dried, and autoradiographed using Kodak BioMax MR film at –70°C for 12 hr.

Identification of Proteins

The labeled viral structural and nonstructural polypeptides were identified by estimation of their molecular weights relative to markers and by comparison with previously reported data [Ericson et al., 1982]. Immunoprecipitations with monoclonal antibodies (MAbs) to rotavirus structural and nonstructural proteins were included to identify unequivocally the corresponding rotavirus proteins. The following MAbs were used: MAb 164AE22 directed against VP2 [Roseto et al., 1983] and MAb ID3 directed against NSP3 [Aponte et al., 1993], kindly supplied by J. Cohen; MAb to VP6 (MAB 851, Chemicon International, Temecula, CA); and MAbs 5A10, 3C3, and 4C3 directed against VP7 [Gerna et al., 1988], obtained from G. Gerna of University of Pavia, Italy. To confirm the identity of VP7 in the RIP assays, radiolabeled proteins extracted from CV-1 cells infected with a recombinant vaccinia virus expressing the VP7 protein of bovine rotavirus strain RF were precipitated with the anti-VP7 monoclonal antibodies. The identification of NSP2 was deduced from the MW of the band corresponding to this protein, previously determined to be 35 K [Aponte et al., 1993], by comparing the patterns of radiolabeled viral proteins electrophoresed on 10% SDS-PAGE and excluding those bands precipitated by MAbs directed against VP7 and NSP3. The NSP2 band could be resolved by SDS-PAGE from VP7 (37 K), which bands between VP6 (44 K) and NSP2, and from NSP3 (34 K). The use of an MAb directed against NSP2 as a marker for this protein was attempted in immunoprecipitation assays. However, it was observed that the MAb LA5 anti-NSP2 [Aponte et al., 1993] coprecipitated other viral proteins (VP1, VP2, VP6, and NSP3) both in high-speed (100,000 g) and low-speed (13,000 g) centrifuged cell lysates.

Statistical Analysis

The two-tailed Student's *t*-test was used to test for the significance of differences between the acute and convalescent antibody responses. To fulfill parametric assumptions, serum antibody titers were log-transformed before the statistical analysis was applied. In all analyses, statistical significance was achieved at the 1% level. Significance, when applied in a statistical comparison, implies a P value of <0.001.

RESULTS

Clinical Features and Diagnosis of Acute Rotavirus Infections

After infants were hospitalized, diarrhea continued for 2–4 days. Watery stools, perianal erythema, and signs of moderate dehydration were recorded in all

TABLE I. Rotavirus-Specific Serum Antibodies (IgM, IgG, IgA) and Coproantibodies (sIgA) Determined by ELISA in Infants With Rotavirus Infection[a]

Patients number (age, month)	Infecting serotype	Sample	Serum IgM	Serum IgG	Serum IgA	Coproantibodies sIgA
1/(2)	G1	1	1/1200	1/1200	1/200	1/50
		2	1/150	1/4800	1/800	1/400
2/(11)	G1	1	1/2400	1/1200	1/50	1/25
		2	1/1200	1/2400	1/100	1/100
3/(14)	G1	1	1/2400	1/1200	1/200	1/100
		2	1/1200	1/9600	1/800	1/400
4/(5)	G3	1	1/2400	1/2400	1/100	1/50
		2	1/1200	1/9600	1/400	1/200
5/(3)	G1	1	1/2400	1/2400	1/50	1/25
		2	1/600	1/4800	1/100	1/100
6/(9)	G1	1	1/4800	1/2400	<1/50	1/25
		2	1/1200	1/4800	1/200	1/50
7/(8)	G1	1	1/9600	1/2400	1/100	1/50
		2	1/4800	1/9600	1/400	1/200
8/(13)	NT	1	1/9600	1/2400	1/100	1/25
		2	1/1200	1/4800	1/400	1/100
9/(24)	G3	1	1/9600	1/600	<1/50	1/25
		2	1/4800	1/4800	1/100	1/100
10/(24)	G1	1	1/4800	1/600	1/100	1/25
		2	1/1200	1/4800	1/400	1/200
11/(7)	G1	1	1/4800	1/1200	<1/50	1/25
		2	1/1200	1/9600	1/100	1/50
Geometric mean titer		1	1/3973[b]	1/1450	1/69	1/34
		2	1/1200	1/5799[b]	1/257[b]	1/137[b]

[a]1 denotes acute phase; 2, convalescent phase; NT, nontypable.
[b]IgM, IgG, IgA, and fecal sIgA were all statistically significant according to the two-tailed Student's *t*-test ($P < 0.001$).

cases. Vomiting and fever were also present. The average duration of diarrhea was 5.8 days.

Rotavirus infections were diagnosed by enzyme immunoassay as described in Materials and Methods and confirmed by visualizing rotavirus particles by electron microscopy in fecal homogenates. No other viruses were found. The presence of bacterial and parasitic enteropathogens were also investigated with negative results.

The results obtained by the serotyping ELISA determined that eight of the children were infected with serotype G1 rotavirus strains and two were infected with serotype G3 strains. One of the field strains was nontypable in our ELISA test.

Antibody Response Assessed by ELISA

The rotavirus-specific antibody responses are shown in Table I. All infants with rotaviral gastroenteritis included in this study showed antirotavirus IgM antibodies during the acute phase of the disease, with titers higher than 1:1,200. Geometric mean titer (GMT) of IgM antibodies detected in acute-phase sera were three times higher than those found during the convalescent phase (1:3,973 vs. 1:1,200) ($P < 0.001$). Only two children showed evidence of high IgM titers (1:4,800) during convalescence. Levels of IgG antibodies in acute-phase sera could be indicative of passively acquired maternal antibodies, very early IgG responses to rotavirus antigens, or, in some children, IgG titers from previous infections. Rotavirus-specific IgG titers detected in the convalescent sera samples were significantly higher (GMT = 1:5,799) than those found during the acute phase with titers of $\geq$1:4,800 in 10 of the 11 children (91%). Serum antirotavirus IgA titers were low in the acute-phase samples: nine children (81.8%) showed levels of $\leq$1:100. In contrast, serum IgA levels rose 3.7 times during convalescence ($P < 0.001$), with titers of $\geq$1:200 in seven children (63.6%).

With regard to the antirotavirus fecal sIgA responses, 10 (91%) samples collected in the acute phase gave titers below or equal to 1:50. Fecal sIgA titers increased four times during the convalescent phase in nine children and showed a geometric mean titer of 1:137 ($P < 0.001$).

Serum IgG Radioimmunoprecipitation Assay

All the sera tested showed reactivity by IgG-RIP assay at 1:10 dilution. The patterns of immunoprecipitated viral polypeptides by paired serum samples from children with natural rotavirus infection are shown in Figure 1. The immunogenicity of viral polypeptides varied slightly between patients but proteins immunoprecipitated with acute and convalescent sera from the same patient remained constant. All serum samples showed strong reactivity against VP6, the main component of the viral inner capsid, and against the nonstructural protein NSP2. In nine patients (81.8%), slight reactivity against VP1 was detected; in seven patients (63.6%), reactivities were noted against VP2 and VP4; and only three sera (27.2%) reacted against the outer-coat glycoprotein VP7. Five serum samples (45.4%) reacted against the nonstructural polypeptides NSP3 and NSP5.

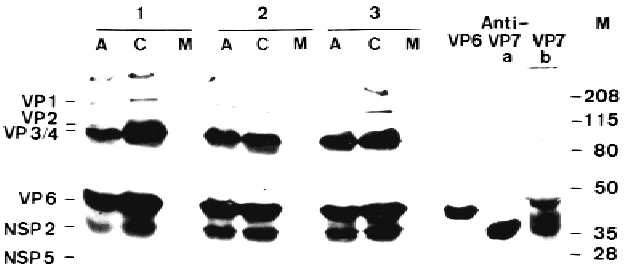

Fig. 1. Immunoprecipitation of [^{35}S]methionine-labeled SA11 rotavirus proteins by IgG antibody in acute-phase (A) and convalescent-phase (C) serum samples collected from three infants with natural rotavirus infections (patients no. 1, 2, and 11, respectively). Mock-infected cell lysates (M) were precipitated by convalescent sera from the same patients. On the right is shown the immunoprecipitation of an SA11 rotavirus-infected cell lysate by MAb to VP6, of (a) a recombinant vaccinia virus expressing VP7 (VV-VP7) cell lysate by MAbs to VP7 and (b) an SA11 rotavirus-infected cell lysate by MAbs to VP7 (run on a different gel). M = molecular weights (in kilodaltons). The immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography. The positions of the viral proteins are shown on the left.

Serum and Fecal IgA Radioimmunoprecipitation Assay

IgA reactivity against rotavirus polypeptides was detected in all sera and convalescent-phase fecal samples. Serum IgA precipitated the structural proteins VP2, VP6, and NSP2 in all samples (Fig. 2). No reactivity was observed against VP4 and some faint bands appeared with VP7 and NSP3. However, no differences in the profiles of the detected proteins were observed among acute- and convalescent-phase serum samples. Convalescent samples showed stronger reactions to the viral polypeptides, correlating with the higher levels of IgA detected by ELISA in these specimens.

Fecal sIgA antibodies in convalescent-phase stool samples reacted very strongly toward NSP2 and with less intensity against VP6 (Fig. 3). Clear responses to VP1 and VP2 were also observed in 5 samples, to VP7 and VP4 in 4 samples, to NSP3 in 8 samples, and to NSP5 in 3 samples. Weaker sIgA reactions were also detected in acute-phase fecal specimens from two patients (18.2%).

Figure 4 shows the main viral polypeptides recognized by convalescent-phase serum samples and by rotavirus-specific fecal antibodies from the 11 infants included in this study. No major differences in the reaction patterns obtained by RIP assay were observed among sera and feces from children infected with rotavirus G1 and G3 serotypes.

DISCUSSION

In order to characterize the specificity of the human IgG and IgA responses to viral polypeptides of naturally infecting rotavirus, we have employed the immunoprecipitation assay using *S. aureus* protein A and the lectin jacalin, respectively. As has been commented by others [Offit, 1994], we consider immunoprecipitation to be the most sensitive procedure for investigating the immune response to individual polypeptides.

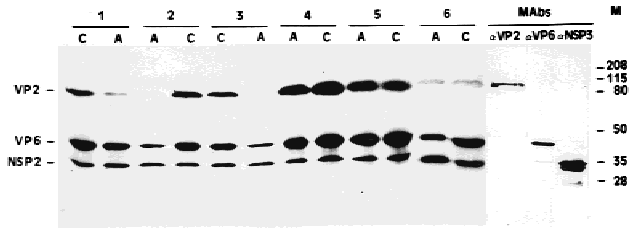

Fig. 2. IgA-immunoprecipitation of rotavirus polypeptides with six paired acute (A) and convalescent (C) serum samples. Numbers indicate serum pairs of patients no. 1 to 6. Immunoprecipitations by MAbs against VP2, VP6, and NSP3 are shown for reference on the right side. M = molecular weights (in kilodaltons).

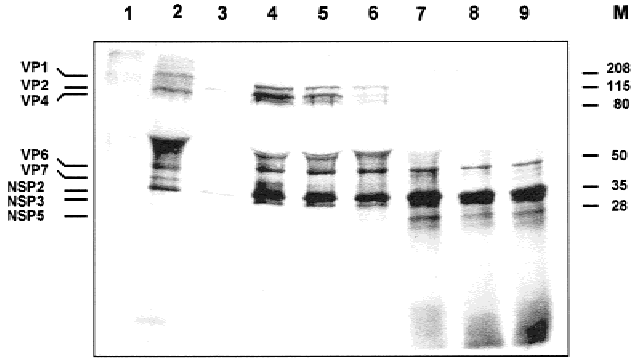

Fig. 3. Fecal sIgA-immunoprecipitation of rotavirus polypeptides by stool specimens from seven children. **Lanes 1** and **2,** IgA-immunoprecipitation with acute- and convalescent-phase fecal samples from patient no. 1; **lanes 3** and **4,** IgA-RIPA with acute- and convalescent-phase fecal samples from patient no. 4; **lanes 5–9,** IgA-RIPA with convalescent-phase stool samples from five infants (patients no. 3, 5, 7, 8, and 9, respectively). M = molecular weights (in kilodaltons).

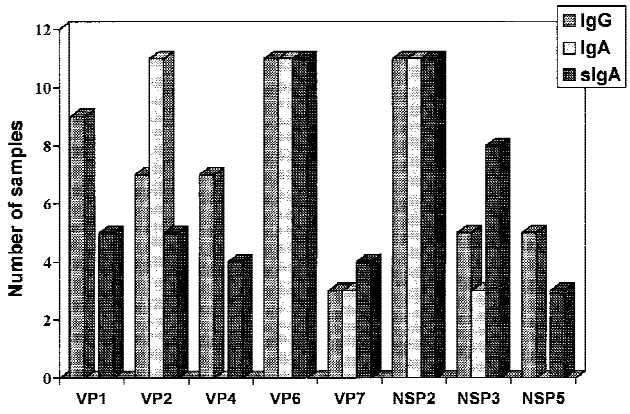

Fig. 4. Number of samples found to be reactive to individual rotavirus polypeptides, showing immune responses to structural and nonstructural viral proteins in convalescent serum samples and stools collected from children (n = 11) with acute rotavirus infection.

Although *S. aureus* protein A does not bind all subclasses of human IgG, this method yields stronger and sharper bands of proteins precipitated by immune sera to rotavirus than those detected by Western blotting.

The detection of serum rotavirus-specific IgG antibodies in acute-phase serum samples may be the consequence of passively acquired maternal antibodies in

some infants, although previous asymptomatic rotavirus infections cannot be excluded. Rotavirus-specific IgM and IgA antibodies were detected in all acute-phase serum samples that were collected within 72 hours after the onset of symptoms. It has been reported that antirotavirus IgM, IgA, and secretory immunoglobulins may appear as early as day 2 after onset [Grimwood et al., 1988]. The absence of serum IgA antibodies has been suggested to be a good marker for the exclusion of previous exposure to rotavirus. We found titers of ⩾1/100 of IgA antibodies in six (54.5%) acute-phase serum samples and rotavirus-specific IgA reactivity by RIPA in all acute serum samples.

The simian rotavirus SA11 has been considered a good surrogate antigen for human rotavirus field strains [Schoub et al.,1977; Kapikian and Chanock, 1996] and has been previously applied in immunoblotting assays to analyze the polypeptides of rotavirus recognized by serum antibodies detected in children [Ushijima et al., 1989; Brüssow et al. 1990; Andrade et al., 1996]. The suitability of this viral strain for use as antigen in ELISA tests, Western blots, and RIP assays for analyzing antibody responses to rotavirus was previously assessed in our laboratory in comparison with the cell-culture-adapted human rotavirus strains Wa and Ito. The results obtained with the serum and fecal samples were very similar regardless of the viral strain used, therefore SA11 was employed as the viral antigen for this study.

The main serum IgG antibody response was directed toward the structural proteins VP2, VP4, and VP6 and the nonstructural protein NSP2. Other viral proteins also detected were VP1, NSP3, and NSP5. Viral protein complexes involving different polypeptides could be immunoprecipitated, some of them corresponding to single-shelled particles [Aponte et al., 1993]. As has also been reported previously, no response to the trypsin cleavage products of VP4, VP5*, and VP8* could be detected [Richardson et al., 1993]. Antibody responses to VP2, VP4, VP6, and NSP2 are of major interest. The VP4 and VP7 proteins independently evoke neutralizing antibodies that are protective in experimental animals [Offit et al., 1986] and in humans [Matsui et al., 1989; Ward et al., 1992].

The viral proteins precipitated by IgG antibodies were the same in both acute- and convalescent-phase sera of the 11 children included in our study, although some differences were detected between the children. The core protein VP1 has been reported to be rarely detected by RIP assays [Ericson et al., 1982; Offit et al., 1983; Johansen et al., 1994]. In our study, 81.8% of sera samples reacted against this protein. It has been suggested that the different reactivities toward this antigen following either natural rotavirus infection or vaccination by the parenteral route may be a consequence of differences in antigen presentation [Svensson et al., 1987].

The inner-core protein VP2 was precipitated by IgG antibody in 63.6% of the sera samples. VP2 has a strong nucleic acid binding activity and, as it forms RNA-protein complexes, is considered to be highly immunogenic [Svensson et al., 1987; Johansen et al., 1994]. This hypothesis is reinforced by the fact that NSP2, a nonspecific RNA-binding protein that accumulates in viroplasms and is required for genome replication, is also highly immunogenic [Svensson et al., 1987], as confirmed in this study. NSP2 elicits not only a strong serum IgG response in rotavirus-infected children but also serum IgA and fecal secretory sIgA responses.

The low IgG and IgA antibody reactivities detected toward VP7, a major neutralizing antigen and the second most abundant protein species in the viral particle [Estes, 1996], are intriguing. Other studies have also shown the absence or low activity of IgG antibodies to VP7 by RIP assay [Svensson et al., 1987; Johansen et al., 1994]. In contrast, several studies in humans indicate that VP7 is the major immunogen after infection [Green et al., 1990; Taniguchi et al., 1991]. However, it has not been determined whether the individual rotavirus polypeptides used in immunoblotting or RIP assays retain the conformation-dependent epitopes present in the virus [Svensson et al., 1987; Taniguchi et al., 1991; Ishida et al., 1996]. In a study conducted by Brüssow et al. [1990] with paired sera from children suffering rotavirus infection, an increase in the titer of antibodies to VP4 was more frequently observed than that to VP7 by the neutralization test and immunoblotting. Using individual baculovirus-expressed rotavirus proteins, Ishida et al. [1996] demonstrated by immunocytochemical staining that adult mice infected with murine rotaviruses predominantly developed IgG immune responses to VP6 and VP4. Weaker responses to VP2, and lesser and delayed responses to VP7, were also observed.

Our results are basically in agreement with the data reported by Svensson et al. (1987), Richardson et al. (1993), and Johansen et al. (1994). Very low and no reactivity against VP4 and VP7 may depend on the infecting P- and G-serotypes and on the viral strain used as antigen source in the RIP assay (SA11 rotavirus in our study). The immune response to VP4, VP7, and NSP4 has been found to be more type-specific. However, low-level antibody responses to VP7 have been reported even when the viral antigen used in the RIP assay was of the same G-specificity as the infecting serotype [Richardson et al., 1993].

With the aim of characterizing the specificity of the serum IgA and fecal sIgA antibody responses, an IgA-RIP assay using jacalin was performed. This lectin is a potent T-cell mitogen and a T-cell-independent activator of human B-cells for the secretion of immunoglobulins. In addition, jacalin binds specifically to the human IgA1 subclass [Roque-Barreira et al., 1985; Kondoh et al., 1986]. Several studies have found that the IgA1 subclass is predominant in both infant sera and in saliva [Fitzsimmons et al., 1994; Friedman et al., 1996]. The IgA-RIP assay performed in this study included jacalin-agarose beads and high salt concentrations in the buffer instead of SDS in order to foster the

detection of antibodies to conformation-dependent epitopes, as previously described [Johansen et al., 1994].

The protective effect of rotavirus VP6-specific IgA monoclonal antibodies lacking neutralizing activity has recently been demonstrated in a murine model. Interestingly, these antibodies were not active in the lumen of the intestinal tract but probably during IgA transcytosis [Burns et al., 1996]. In a study performed to evaluate the efficacy of DNA vaccines encoding murine rotavirus VP4, VP6, and VP7 in adult mice challenged with the homotypic rotavirus strain EDIM, the highest levels of rotavirus-specific fecal IgA antibody were observed in those mice inoculated with the VP6 DNA vaccine [Herrmann et al., 1996]. Although each of the three DNA vaccines provided protective immunity as measured by reduction in viral shedding, no neutralizing antibodies were detectable in mice given the plasmid encoding VP6. It is therefore possible that IgA-mediated intracellular neutralization may be responsible for the protection induced with this VP6 DNA vaccine. It has been proposed that IgA is transported through epithelial cells by the polymeric immunoglobulin receptor and may interfere with viral replication by binding to newly synthesized viral proteins within infected cells, thereby neutralizing microbial pathogens intracellularly [Mazanec et al., 1992]. The capacity of IgA to abort virus replication within epithelial cells has already been demonstrated for influenza virus [Mazanec et al. 1995].

The results of our study demonstrate that, after rotavirus infection, serum IgA antibodies are predominantly directed against VP2, VP6, and NSP2. Interestingly, VP4-specific IgA antibodies could not be detected in any serum sample. The same result was previously reported by Johansen et al. (1994). The absence of reactivity to this protein could be the consequence of differences in avidity of IgA1 subclass antibodies. In our study, fecal sIgA major responses to VP6 and to NSP2 have been detected by IgA-RIPA for the first time. Weak reactions were also detected toward other structural (VP1 and VP2) and nonstructural proteins (NSP3 and NSP5). We consider these findings interesting with regard to the role that sIgA may play in the protection against rotavirus infections.

Most studies on antibody protection against rotavirus diarrhea in children have focused on the neutralizing antibodies to VP4 and VP7 [Matsui et al., 1989; Green et al., 1990; O'Ryan et al., 1994]. However, other viral proteins elicit antibody responses that may play important roles in the resolution and protection against rotavirus infection and disease. It has been recently reported that the immune response to VP6 in feces seems to be the best predictor of protection after heterologous immunization in a mouse model [Feng et al., 1997]. The results obtained evaluating the efficacy of rotavirus DNA vaccines in mice give greater substance to this observation [Herrmann et al., 1996]. The high reactivity of fecal sIgA toward VP6 detected in this study in infants with rotavirus infection provides further confirmation of those results and implies the same immune response in humans.

ACKNOWLEDGMENTS

We thank J. Cohen for providing the monoclonal antibodies to rotavirus VP2, NSP2, and NSP3 and the recombinant vaccinia virus VV-VP7; G. Gerna for supplying the monoclonal antibodies to VP7; and A.P. MacCabe and D. Navarro for critical reviewing of the manuscript.

REFERENCES

Andrade GP, Lima LRAV, Hoshino-Shimizu S, Oliveira MI, Mendonça RZ, Hársi CM, Stewien KE (1996): Humoral immunity patterns based on antibody reactivity to rotavirus antigens in Brazilian children under 5 years of age. Journal of Medical Virology 49:212–217.

Aponte C, Mattion MN, Estes MK, Charpilienne A, Cohen J (1993) Expression of two bovine rotavirus nonstructural proteins (NSP2, NSP3) in the baculovirus system and production of monoclonal antibodies directed against the expressed proteins. Archives of Virology 133:85–95.

Brüssow H, Offit PA, Gerna G, Bruttin A, Sidoti J (1990): Polypeptide specificity of antiviral serum antibodies in children naturally infected with human rotavirus. Journal of Virology 64:4130–4136.

Burns JW, Siadat-Pajouh M, Krishnaney AA, Greenberg HB (1996): Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. Science 272:104–107.

Coulson BS, Unicomb LE, Pitson GA, Bishop RF (1987): Simple and specific enzyme immunoassay using monoclonal antibodies for serotyping human rotaviruses. Journal of Clinical Microbiology 25: 509–515.

Coulson BS, Grimwood K, Bishop RF, Barnes GL (1989): Evaluation of end-point titration, single dilution and capture enzyme immunoassays for measurement of antirotaviral IgA and IgM in infantile secretions and serum. Journal of Virological Methods 53:53–66.

Ericson BL, Graham DY, Mason BB, Estes MK (1982): Identification, synthesis, and modifications of simian rotavirus SA11 polypeptides in infected cells. Journal of Virology 42:825–839.

Estes MK (1996): Rotaviruses and their replication. In Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizman B, Straus SE (eds): "Fields Virology," 3rd edit. Philadelphia: Lippincott-Raven, pp 1625–1655.

Feng N, Burns JW, Bracy L, Greenberg H (1994): Comparison of mucosal and systemic humoral immune responses and subsequent protection in mice orally inoculated with a homologous and a heterologous rotavirus. Journal of Virology 68:7766–7773.

Feng N, Vo PT, Chung D, Vo T-VP, Hoshino Y, Greenberg H (1997): Heterotypic protection following oral immunization with live heterologous rotaviruses in a mouse model. Journal of Infectious Diseases 175:330–341.

Fitzsimmons SP, Evans MK, Pearce CL, Sheridan MJ, Weintzen R, Cole MF (1994): Immunoglobulin A subclasses in infants' saliva and in saliva and milk from their mothers. Journal of Pediatrics 124:566–573.

Friedman MG, Entin N, Zedaka R, Dagan R (1996): Subclasses of IgA antibodies in serum and saliva samples of newborns and infants immunized against rotavirus. Clinical and Experimental Immunology 103:206–211.

Gerna G, Sarasini A, Matteo AD, Passarani N, Gagliardi V, Milanesi G, Astaldi-Ricotti G, Battaglia M (1988): The outer capsid glycoprotein VP7 of simian rotavirus SA11 contains two distinct neutralization epitopes. Journal of General Virology 69:937–944.

Glass RI, Gentsch J, Smith JC (1994): Rotavirus vaccines: Success by reassortment? Science 265:1389–1391.

Green KY, Taniguchi K, Mackow ER, Kapikian AZ (1990): Homotypic and heterotypic epitope-specific antibody responses in adult and infant rotavirus vaccines: Implications for vaccine development. Journal of Infectious Diseases 161:667–669.

Grimwood K, Lund JC, Coulson BS, Hudson IL, Bishop RF, Barnes GL (1988): Comparison of serum and mucosal antibody responses

following severe acute rotavirus gastroenteritis in young children. Journal of Clinical Microbiology 26:732–738.

Herrmann JE, Chen SH, Fynan EF, Santoro JC, Greenberg HB, Wang S, Robinson HL (1996): Protection against rotavirus infections by DNA vaccination. Journal of Infectious Diseases 174(suppl 1):S93–S97.

Hjelt K, Grauballe PC, Paerregaard A, Nielson OH, Krasilnikoff PA (1987): Protective effect of preexisting rotavirus-specific immunoglobulin A against naturally acquired rotavirus infection in children. Journal of Medical Virology 21:39–47.

Inouye S, Matsuno S, Yamaguchi H (1984): Efficient coating of the solid phase with rotavirus antigens for enzyme-linked immunosorbent assay of immunoglobulin A antibody in feces. Journal of Clinical Microbiology 19:259–263.

Ishida SI, Feng N, Tang B, Gilber JM, Greenberg HB (1996): Quantification of systemic and local immune responses to individual rotavirus proteins during rotavirus infection in mice. Journal of Clinical Microbiology 34:1694–1700.

Johansen K, Granqvist L, Karlen K, Stintzing G, Uhnoo I, Svensson L (1994): Serum IgA immune response to individual rotavirus polypeptides in young children with rotavirus infection. Archives of Virology 138:247–259.

Kapikian AZ, Chanock RM (1996): Rotaviruses. In Fields BN, Knipe DN, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizman B, Straus SE (eds): "Fields Virology," 3rd edit. Philadelphia: Lippincott-Raven, pp 1657–1708.

Kondoh H, Kobayshi K, Hagiwara K, Kajii T (1986): Jacalin, a jackfruit lectin, precipitates IgA1 but not IgA2 subclass on gel diffusion reaction. Journal of Immunological Methods 88:171–173.

Matsui SM, Offit PA, Vo PT, Mackow ER, Benfield DA, Shaw RD, Padilla-Noriega L, Greenberg HB (1989): Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to the heterotypic neutralization domain of VP7 and the VP8 fragment of VP4. Journal of Clinical Microbiology 27:780–782.

Mazanec MB, Kaetzel CS, Lamm ME, Fletcher D, Nedrud JG (1992): Intracellular neutralization of virus by immunoglobulin A antibodies. Proceedings of the National Academy of Science U.S.A. 89:6901–6905.

Mazanec MB, Coudret CL, Fletcher DR (1995): Intracellular neutralization of influenza virus by immunoglobulin A antihemagglutinin monoclonal antibodies. Journal of Virology 69:1339–1343.

Midthun K, Kapikian AZ (1996): Rotavirus vaccines: An overview. Clinical Microbiology Reviews 9:423–434.

O'Ryan ML, Matson DO, Estes MK, Pickering LK (1994): Antirotavirus G-type-specific and isotype-specific antibodies in children with natural rotavirus infections. Journal of Infectious Diseases 169: 504–511.

Offit PA, Clark HF, Plotkin SA (1983): Response of mice to rotavirus of bovine or primate origin assessed by radioimmunoassay, radioimmunoprecipitation, and plaque reduction neutralization. Infection and Immunity 42:293–300.

Offit PA, Clark HF (1985): Protection against rotavirus-induced gastroenteritis in a murine model by passively acquired gastrointestinal but not circulating antibodies. Journal of Virology 54:58–64.

Offit PA, Shaw RD, Greenberg HB (1986): Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to surface proteins VP3 and VP7. Journal of Virology 58:700–703.

Offit PA (1994): Immunologic determinants of protection against rotavirus disease. In Ramig RF (ed): "Rotaviruses." New York: Springer-Verlag, pp 229–254.

Richardson SC, Grimwood K, Bishop RF (1993): Analysis of homotypic and heterotypic serum immune response to rotavirus proteins following primary rotavirus infection by using the radioimmunoprecipitation technique. Journal of Clinical Microbiology 31:377–385.

Roque-Barreira MC, Campos-Nieto A (1985): Jacalin: An IgA-binding lectin. Journal of Immunology 134:1740–1743.

Roseto A, Scherrer R, Cohen J, Guillemin MC, Charpilienne A, Feynerol C, Peries J (1983): Isolation and characterization of antirotavirus immunoglobulins secreted by cloned hybridoma cell lines. Journal of General Virology 64:237–240.

Schoub BD, Lecatsas G, Prozesky OW (1977): Antigenic relationship between human and simian rotaviruses. Journal of Medical Microbiology 10:1–7.

Shaw RD, Merchant AA, Groene WS, Cheng EH (1993): Persistence of intestinal antibody response to heterologus rotavirus infection in a murine model beyond 1 year. Journal of Clinical Microbiology 31: 188–191.

Svensson L, Sheshberadaran H, Vene S, Norrby E, Grandien M, Wadell G (1987): Serum antibody responses to individual viral polypeptides in human rotavirus infection. Journal of General Virology 68:643–651.

Taniguchi K, Urasawa T, Kobayashi N, Ahmed MV, Adachi N, Chiba S, Urasawa S (1991): Antibody response to serotype-specific and cross-reactive neutralization epitopes on VP4 and VP7 after rotavirus infection or vaccination. Journal of Clinical Microbiology 29: 483–487.

Ushijima H, Honma H, Ohnoda HA, Mukojama J, Oyanagi HY, Araki K, Shinozaki T, Morikawa S, Kitamura T (1989): Detection of antirotavirus IgG, IgM, and IgA antibodies in healthy subjects, rotavirus infections, and immunodeficiencies by immunoblotting. Journal of Medical Virology 27:13–18.

Ward RL, Clemens JD, Knowlton DR, Rao MR, van Loon FP, Huda N, Ahmed F, Schiff GM, Sack DA (1992): Evidence that protection against rotavirus diarrhea after natural infection is not dependent on serotype-specific neutralizing antibody. Journal of Infectious Diseases 166:1251–1257.

Ward RL (1996): Mechanisms of protection against rotavirus in humans and mice. Journal of Infectious Diseases 174(Suppl 1):S51–S58.